Journal of Surgical Oncology 1997;66:39–44

Expression of ICAM-1 Enhances In Vivo Lymphocyte Adhesion in a Murine Fibrosarcoma

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Background and Objectives: ICAM-1 is essential for lymphocyte-endothelial cell interactions. We have demonstrated that increased expression of ICAM-1 in tumors results in an enhanced response to adoptive immunotherapy. We undertook this study to determine whether increased expression of ICAM-1 results in increased lymphocyte adhesion in vivo.
Methods: Parental MCA-105 tumor cells were cotransfected with ICAM-1 and the NeoR plasmid. A neomycin resistant clone (Cl149) was selected and increased expression of ICAM-1 confirmed by FACS analysis. Tumor fragments (MCA-105 or Cl149) were placed in a dorsal skinfold chamber on day 0 in C57BL/6 mice. Lymphocytes were fluorescently labeled using 0.5% acridine orange and activity recorded on videotape at 700× magnification. Lymphocyte activity was quantitated over 30 second intervals in postcapillary venules as either passing or rolling/sticking (R/S). The % R/S was calculated for each category and evaluated using χ^2 analysis.
Results: Whereas 38% of lymphocytes were classified as R/S in normal tissue, 32% were classified as R/S ($P > .05$) in the MCA-105 tumor. However, in the ICAM-1 transfected CL149, there was significantly greater R/S at 53% ($P < .05$).
Conclusions: These data demonstrate increased lymphocyte adhesion in tumors with enhanced expression of ICAM-1 by direct in vivo observations and may partially explain the salutary effect of increased ICAM-1 expression on adoptive immunotherapy. This suggests the possible application of adhesion molecule expression in the cellular therapy of cancer.
J. Surg. Oncol. 1977;66:39–44.

KEY WORDS: ICAM-1; lymphocyte adhesion; murine tumor; video microscopy

INTRODUCTION

The cellular immunotherapy of cancer has received a great deal of attention, but therapeutic results have not met the expectations that arose from in vitro studies. Cellular therapy of cancer is limited by the ability of the effector cells to gain access to the tumor. The mechanism of action of cytotoxic cells is unclear, but may be due to extravasation of effector cells resulting in direct cell-cell contract between effector cells and target cells, which mimics in vitro experiments. Alternatively, effector cells may release of cytokines in the vicinity of target cells, or there may be an effect of effector cells on tumor vasculature leading to infarction. The ability of effector cells to

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Accepted 6 June 1997

get to the tumor cells may not be sufficient for destruction of the target cells. If direct contact is required for target cell destruction, the ability of the effector cell to lyse the tumor cell is dependent on its ability to leave the vasculature. In order for effector cells to leave the vessel lumen, they must first adhere to the vascular endothelial cells.

Intercellular adhesion molecule-1 (ICAM-1) is a member of the family of integrins and can be found on the surface of vascular endothelial cells. ICAM-1 acts as the primary ligand for lymphocyte function-associated antigen-1 (LFA-1), which is expressed on the surface of lymphocytes. ICAM-1 has been shown to play a major role in lymphocyte adhesion at the endothelial cell layer [1]. In order directly to modulate lymphocyte adhesion, we transfected the human ICAM-1 gene into the MCA-105 murine fibrosarcoma.

We have previously reported that ICAM-1 transfectants have increased ICAM-1 expression in vitro and have a reduced tumor growth rate in vivo compared to parental tumor in untreated animals or animals treated with hyperthermia and IL-2 [2]. Although, in vitro experiments by others have shown no increased adhesion between murine lymphocytes and human ICAM-1 [3], our own in vivo results have shown significantly decreased growth rate of murine tumors transfected with human ICAM-1 compared with parental tumor cells or cells transfected with the neomycin-resistance gene only [2]. Therefore, we hypothesized that the reduced growth rate of ICAM-1 expressing tumors may be secondary to increased in vivo lymphocyte adhesion. The increased adhesion has been observed in our laboratory using histologic sections of resected tumor [4]. However, it is difficult to assess how accurately this represents lymphocyte activity in the live tumor.

A number of mechanisms can explain the slowed growth of ICAM-1 expressing tumors. It may be due to an immune response to the human ICAM-1 in a murine model. Recent data from our laboratory using MCA-105 tumors transfected with murine ICAM-1 have shown similar results to that observed with human ICAM-1 (unpublished data), suggesting that this is an unlikely explanation. Alternatively, ICAM-1 may lead to increased T-cell activation in the local tumor environment. A third possible mechanism may be due to increased lymphocyte adhesion secondary to an increase in the available binding sites. Naturally, the observed effect may result from a combination of these possible mechanisms. We undertook these experiments to observe and quantitate lymphocyte activity in the vessels of normal tissue compared to vessels of tumor tissue in a model that includes the dynamics of a living tumor.

MATERIALS AND METHODS

Animal Care

Ex-breeder female C57BL/6 mice (Charles River, Wilmington, MA) were given routine care following NIH guidelines. Surgical procedures for implantation of the tissue chamber and all experiments described herein were fully approved by the University of Maryland at Baltimore Institutional Animal Care and Use Committee (IACUC) prior to undertaking this work.

Tumors

MCA-105, a methylcholanthrene-induced fibrosarcoma syngeneic to C57BL/6 mice (kindly supplied by Dr. S. Rosenberg, Bethesda, MD), was used as the parental cell line. Clone 149, cotransfected with the human ICAM-1 and neomycin resistance (NeoR) genes, was used as the source of ICAM-1 expressing tumors.

ICAM-1 Gene Transfections

This work was performed as previously described by Sartor [4]. Briefly, the plasmid encoding for functional ICAM-1 (generously provided by Dr. Timothy Springer, Harvard Medical School, Boston, MA) was isolated, purified, and then transfected into the MCA-105 cell line along with the gene for neomycin resistance using the calcium phosphate technique. G418 resistant clones were then selected and gene incorporation verified by Southern blot DNA analysis. Transcription of the ICAM-1 gene was verified by Northern blot analysis of mRNA. ICAM-1 gene expression was confirmed by FACS analysis, and a high expressing clone (Clone 149) was selected for further study. ELISA analysis showed significantly greater amounts of ICAM-1 released into the supernatant by transfectants (0.02 ng/ml) compared to parental tumor cells (0.0 ng/ml).

In Vivo Tumor Model

Mice, anesthetized with pentobarbitol 40 mg/kg by intraperitoneal injection, were fitted with a dorsal skinfold chamber (obtained from Dr. L.M. Messmer, Munich, Germany) on day 0 using a modified technique from that originally described by Papenfus et al. [5]. Viable tumor fragments were placed into the chamber on day 0 and allowed to grow from 4 to 6 days. On the day of video microscopy, intravenous access was established via cannulation of the inferior vena cava with size 10 polyethylene tubing. The cannula was connected to a syringe containing a 0.5% acridine orange solution (Sigma, St. Louis, MO) for the eventual fluorescence of lymphocytes [6]. This model has been extensively characterized by Wu and coworkers [6] and accurately identifies lymphocyte activity. In addition, an intraperitoneal catheter was placed for the further administration of pentobarbitol anesthesia. Next, the coverslide of the tissue

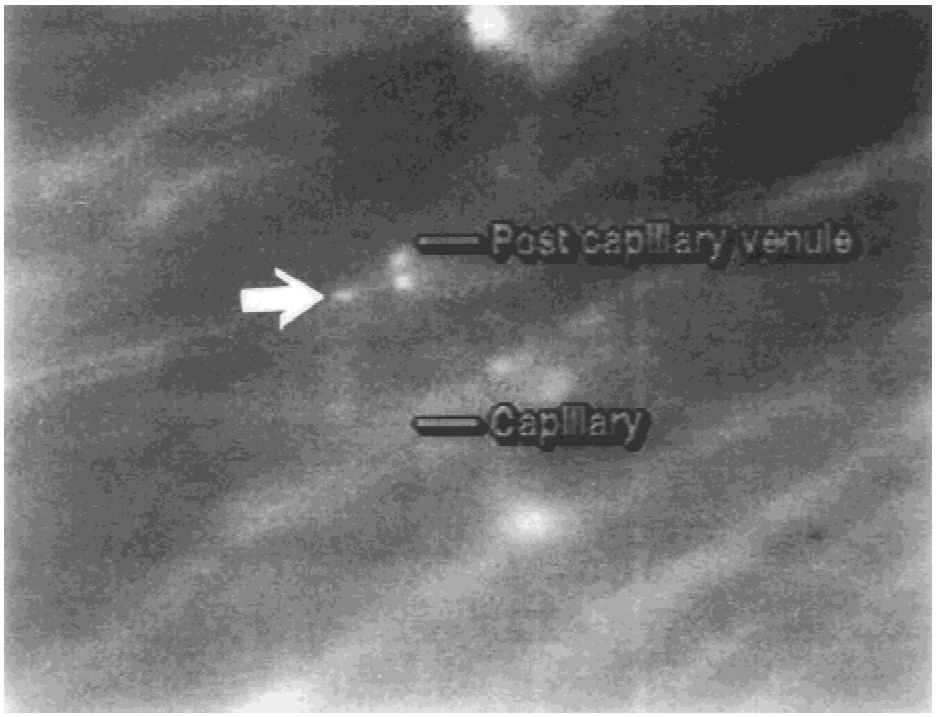

Fig. 1. A representative image from the video microscopy system is shown here with a postcapillary venule and capillary identified. A fluorescently labeled cell is identified (arrow).

chamber was removed and the membranes overlying the vascular bed were cleared away using microsurgical technique. The chamber was then filled with normal saline to protect the vascular bed from dessication. In four of the animals TNFα (500 units/cc) was applied topically to the vascular bed and allowed to incubate for 4 hours prior to video microscopy.

Video Microscopy

Mice were immobilized gently on a plexiglass holder attached to a temperature-controlled microscope stage. Using a reflected fluorescence microscope (Olympus BH-2, Tokyo, Japan), acridine orange was excited at 490 nm and emission at 500 nm observed with a barrier filter. Fluoresced light was enhanced by the use of a Dark Invader Night Version System (Meyers & Co., Redmond, WA) with 700× magnification for image recording. Postcapillary venules were identified, and injections of 0.03 cc of 0.5% acridine orange solution over 10 seconds were used to fluoresce lymphocytes. The lymphocyte activity was recorded on video tape for future analysis. Analysis consisted of observation of a postcapillary venule for a 30-second interval beginning after the appearance of the first fluorescently labeled lymphocyte. Lymphocyte activity was classified as either passing or rolling/sticking. A passing lymphocyte was defined as one that showed no interruption in its flow in relation to the surrounding erythrocyte flow. A rolling/sticking lymphocyte was defined as one that did show an interruption in its flow in relation to the surrounding erythrocyte flow.

Statistical Analysis

The total number of lymphocytes were counted and the ratio for rolling/sticking was calculated. These values were compared and analyzed using the chi squared test (SYSTAT Software, Evanston, IL) on an IBM (Armonk, NY) microcomputer.

RESULTS

Combination of Fluorescence Video Microscopy and Dorsal Skinfold Chamber in C57BL/6 Mice Allows for Accurate Measurements of In Vivo Lymphocyte Adhesion

Prior in vitro studies in our laboratory indicated that lymphocyte adhesion is modified by ICAM-1 expression [7]. Histologic studies of tumors transfected with ICAM-1 have shown an increased lymphocytic infiltrate [4]. However, the effect on lymphocyte adhesion in living tissue may not be represented by static images or in vitro studies. We therefore designed this system to investigate the in vivo effect of ICAM-1 on lymphocyte adhesion. Fluorescence video microscopy has been used in the past to study the activity of lymphocytes in normal

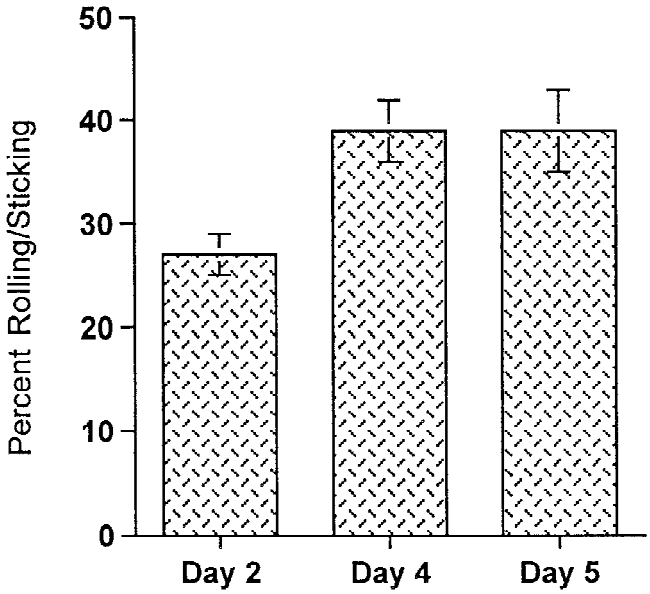

Fig. 2. Comparison of lymphocyte adhesion according to time following chamber implantation revealed that chambers 2 days old had less ($P = .053$) adhesion than chambers 4 and 5 days old. Error bars indicate standard error of the mean (±SEM) of percent lymphocytes rolling/sticking.

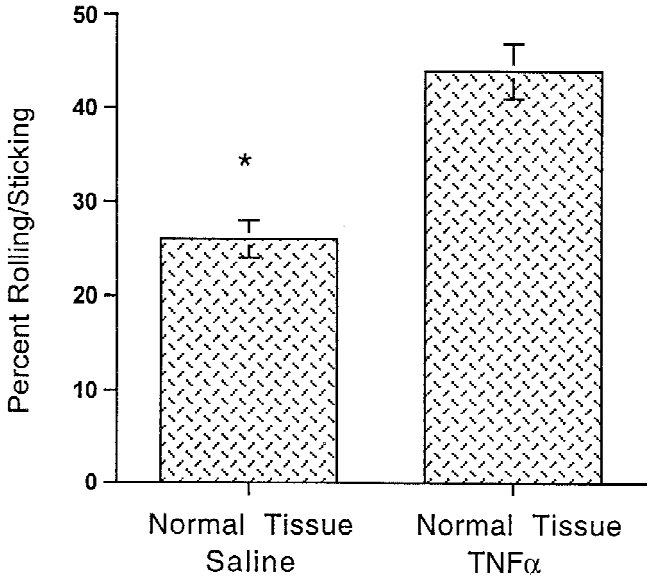

Fig. 3. Lymphocytes showed significantly less ($P < .03$, indicated by *) adhesion to normal tissues bathed in saline compared to normal tissues bathed in TNFα (500 U/cc) for 4 hr prior to in vivo adhesion measurement using the dorsal skinfold chamber model. Error bars indicate standard error of the mean (±SEM) of percent lymphocytes rolling/sticking.

as well as tumor vasculature. The dorsal skinfold chamber also has been used as a tool for the investigation of lymphocyte activity in the rat, hamster, and SCID mouse. A representative video image is shown in Figure 1. In this experiment, three chambers were observed at 2 days, four chambers were observed at 4 days, and five chambers were observed at 5 days. The lymphocyte activity was nearly significantly less ($P = .053$) for 26% rolling/sticking in 2-day-old chambers in 15 separate observations, compared with 39% rolling/sticking in 4- and 5-day-old chambers (Fig. 2) in 12 and 23 observations, respectively. Further comparisons were conducted with chambers at the same time point following implantation. These experiments were repeated with similar results.

TNFα Significantly Increases Lymphocyte Adhesion in Dorsal Skinfold Chambers

TNFα has been shown to increase lymphocyte adhesion. We therefore wanted to determine whether the addition of TNFα would increase adhesion in this model as a positive control. TNFα was used to treat four animals with 2-day-old chambers, which resulted in 44% lymphocyte rolling/sticking in 21 separate observations. Comparison of lymphocyte adhesion in the TNFα-treated animals to that in 2-day-old chambers with untreated normal tissue (26%) revealed significantly ($P < .05$) increased adhesion in animals treated with TNFα, confirming the effect of TNFα in this model. Representative data are shown in Figure 3. These experiments were repeated with similar results.

Lymphocyte Adhesion to Parental Tumor Vessels and Normal Tissue Vessels Is Similar

Lymphocyte-endothelium interaction in vivo is comprised of passing, rolling, and adhering. Prior studies in our laboratory determined that the MCA-105 parental tumor line does not express ICAM-1. This experiment was designed to determine if there was a difference in lymphocyte adherence between normal tissues and MCA-105, both being tissues that do not express ICAM-1. For this experiment, 11 animals were fitted with the dorsal skinfold chamber for normal tissue observation, and nine animals contained chambers with MCA-105 tumor. Lymphocyte adherence in normal tissue was 40% in 35 separate observations as measured by the percent of rolling/sticking of fluorescently labeled lymphocytes in a postcapillary venule. In MCA-105 tumors, 38% of lymphocytes were adherent to the tumor-affected vessels. The statistical comparison of these two values ($P = .67$), shows no significant difference in adhesion. These experiments were repeated with similar results.

Lymphocyte Adhesion Is Significantly Greater in ICAM-1 Expressing Tumors Compared to Parental Tumors

ICAM-1 is a major ligand for lymphocyte adhesion as determined in in vitro studies. This experiment was de-

signed to determine if, in the in vivo environment, the increased expression of ICAM-1 in tumor cells transfected with the ICAM-1 gene, showing expression of ICAM-1, would increase lymphocyte adhesion. Six animals were fitted with the dorsal skinfold chamber and implanted with a clone 149 (high ICAM-1 expressor) tumor fragment. The percent rolling/sticking in the tumor with high expression of ICAM-1 was 53% in 41 separate observations. This value was significantly ($P < .03$) greater than the percent rolling/sticking in vessels of normal tissues (39%) and parental tumors (32%). These experiments were repeated with similar results. This study demonstrates in living tissue that transfection of a murine tumor with the human ICAM-1 gene does in fact increase lymphocyte adhesion to postcapillary venules. These data suggest that the improved response to adoptive immunotherapy seen in these tumors may be a result of increased delivery of effector cells through increased adhesion.

DISCUSSION

Lymphocyte adhesion is an essential step in the process of successful cellular therapy for cancer. The more efficiently effector cells can adhere to vascular endothelium and subsequently leave the vasculature, the greater number can reach and eventually lyse the tumor. Since prior studies have indicated that ICAM-1 plays a major role in lymphocyte-endothelial interactions [8], our laboratory believed that transfecting tumor cells with ICAM-1 would increase lymphocyte adhesion and subsequently enhance cellular therapy. Studies investigating the in vivo growth of the ICAM-1 transfected tumors revealed a spectrum of growth rates proportional to ICAM-1 expression. ICAM-1 expressing tumors grew more slowly in untreated animals than the parental tumor [4]. In addition, the tumors with high expression of ICAM-1 grew more slowly than tumors with low expression of ICAM-1 [4]. Further studies in animals treated with IL-2 and hyperthemia showed similar findings of slowed growth in ICAM-1 expressing tumors [2]. Additional in vitro studies revealed that the ICAM-1 transfected tumors also secreted soluble ICAM-1 [8]. Histologic studies of ICAM-1 expressing tumors compared to parental tumor were significant for the increased presence of lymphocytes in the ICAM-1 expressing tumor [4]. Based on these histologic findings, we hypothesized three possible mechanisms, which are not mutually exclusive, to explain the slowed growth rate in the ICAM-1 expressing tumors.

The first hypothesis is that ICAM-1 causes an increase in T-cell activation in the local tumor environment. Studies have investigated the possible interaction between the T-cell receptor and ICAM-1 binding. Van Seventer et al. [9] showed that the ICAM-1/lymphocyte function-associated antigen-1 (LFA-1) interaction, through adhesion and signal transduction, acts as a costimulus in the activation of T-cells. In addition, Braakman et al. [10] demonstrated that the ICAM-1/LFA-1 binding is involved in T-cell activation through promoting adhesion of cytotoxic T lymphocytes to target cells and by producing co-stimulatory signals to the T-cell receptor in cell-mediated lysis. These studies imply that enhancing ICAM-1 related adhesion may lead to an improved efficacy in cellular therapy. Furthermore, the ability of ICAM-1 to stimulate T cells may be a mechanism whereby increased ICAM-1 production by tumor cells increases the efficacy of cellular immunotherapy.

The second hypothesis for the role of ICAM-1 may be explained through the interaction of a human gene expressed in murine tissue. The slowed in vivo growth and the histologic findings of increased lymphocytes could be secondary to an increased immune response to the foreign gene. Xu et al. [11] have demonstrated that human ICAM-1 possesses a 53% structural homology with murine ICAM-1. Functionally, however, others have reported that species restriction exists. Johnston et al. [3] has shown that in an in vitro environment, murine LFA-1 does not bind to human ICAM-1. However, recent in vivo data from our laboratory demonstrates that tumors transfected with murine ICAM-1 respond similarly to those transfected with human ICAM-1, yet significantly different from parental tumor or tumor cells transfected with G418 alone (unpubl. data). The current model shows that murine tumors transfected with human ICAM-1 affect lymphocyte trafficking in vivo as there was significantly increased adhesion seen in the vessels of the ICAM-1 expressing tumors.

The third hypothesis to explain the effect of increased ICAM-1 expression may be related to its role in adhesion. There may be increased lymphocyte binding and eventual tumor cell lysis secondary to a greater number of binding sites present. In vitro studies in our laboratory have shown that ICAM-1 expressing tumor cells exhibit greater lymphocyte adhesion and increased LAK cell mediated cytotoxicity [7]. The mechanism by which increased tumor cell expression of ICAM-1 enhances lymphocyte adhesion to endothelial cells may be related to a ''bystander effect'' with increased levels of ICAM-1 in the interstitial space. Until now, there have been no in vivo studies done to evaluate lymphocyte adhesion in vessels affected by ICAM-1.

In order to evaluate the in vivo effects of ICAM-1 on lymphocyte adhesion, an accurate system for observation as well as quantification of lymphocyte-endothelial interactions was constructed. The murine dorsal skinfold chamber has been used to grow tumor tissue in other studies [12,13], and fluorescence enhanced video microscopy has been used for in vivo lymphocyte observation. But our system is unique in that it allows quantification of lymphocyte-endothelial activity in normal as well as

tumor tissue in immunocompetent mice. In addition, the ability directly to observe lymphocyte-endothelial activity in ICAM-1 expressing tumors is unique to our laboratory. Although it was previously thought that rolling/sticking occurred only in inflammatory states, it has been subsequently shown that rolling and sticking occurs in normal noninflamed vessels [14]. The 39% lymphocyte rolling/sticking observed in our tissue chambers for normal tissue is within the range reported by Mayrovitz [14] for lymphocyte activity in nonoperative fields. In addition, the sensitivity of the system was tested by adding TNFα to the tissue chamber. TNFα is known to stimulate the expression of endothelial leukocyte adhesion molecule 1 (ELAM-1) and the endothelial ligand for leukocyte adhesion molecule 1 (L-selectin) [15]. The observed values for % rolling/sticking of 27% and 44% ($P < 0.05$) (as shown in Fig. 3) for normal and TNFα treated tissues respectively are similar to results reported by Wu [6].

The comparison of % lymphocyte rolling/sticking for normal tissue to that of non-ICAM-1 expressing tumor, although not statistically significant in these experiments ($P = .67$), is important. The % rolling/sticking in parental tumor tissue establishes a baseline value for comparison of lymphocyte adhesion in ICAM-1 expressing clones, as the only difference between the parental cell line and ICAM-1 expressing clones is the presence of ICAM-1.

The value obtained for lymphocyte adhesion in ICAM-1 expressing tumor tissue, 53%, is significantly greater ($P < .03$) than the values obtained for normal and parental tumor tissue. Tumor-affected vessels are characteristically more tortuous than normal vessels. But as seen in the parental tumor, the change in morphology does not lead to increased lymphocyte adhesion. Since the vessels observed are of host etiology, a mechanism must exist to link ICAM-1 expression on tumor cells to increased adhesion to the endothelium of local vessels. It has been shown that ICAM-1 expression clones secrete soluble ICAM-1 [4]. It is possible that this soluble ICAM-1 leads to the increased adhesion of lymphocytes to the endothelial cells of nearby postcapillary venules through one or a combination of the mechanisms previously described.

CONCLUSIONS

The experiments described here demonstrate that there is increased leukocyte-endothelial adhesion in the vessels of tumors with enhanced ICAM-1 expression. This increased adhesion may play a role in explaining the slowed in vivo growth characteristics of ICAM-1 expressing tumors, as well as their increased response to adoptive immunotherapy. Future studies involving the tissue chamber model will include the evaluation of IL-2 immunotherapy and hyperthermia, which have already been shown in our laboratory to have an enhanced effect on tumors expressing ICAM-1 compared to tumors not expressing ICAM-1 [2]. The use of ICAM-1 and other adhesion molecules may hold promise as targets for in vivo gene therapy in the enhancement of cellular therapy in cancer treatment.

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